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Changes of polyamine pattern in digestive glands of mussel *Mytilus galloprovincialis* under exposure to cadmium

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ABSTRACT

Polyamines, in particular spermidine and spermine, have been identified as important antioxidants, highly induced by oxidative stress in a variety of organisms. However, little is known about changes in polyamine content of metal-stressed marine organisms. In the present study, mussels (*Mytilus galloprovincialis*) were experimentally exposed to 25 μg/L Cd^{2+} or 100 μg/L Cd^{2+} for up to 15 days. Cd^{2+} was progressively accumulated in mussel tissues, leading to a characteristic oxidative-stress status. Free putrescine (PUT) production was noticeably induced in response to Cd^{2+} at day 5 and then declined. In contrast, free spermidine (SPD) content was gradually reduced, whereas the concentration of free spermine (SPM) increased. In combination, these changes led to a 69% or 88% reduction in the ratio of (SPD + SPM)/PUT at day 5, dependent on the Cd^{2+} concentration used, which subsequently followed an upward trend in values, albeit not reaching those of controls. Conjugated polyamines constantly increased, in particular conjugated spermidine and spermine, tagging along with metallothionein production. Acetylated polyamines showed a diverse profile of changes, but their content was generally kept at low levels throughout the exposure period. Collectively, our results suggest that certain polyamine compounds could play a significant role in the tolerance of mussels against Cd^{2+}-mediated stress, and that the ratio (SPD + SPM)/PUT could be a good indicator of the metal-stress status.

1. Introduction

Polyamines (PAs) are polycationic biogenic amines required for both prokaryotic and eukaryotic cell growth and differentiation (Cohen, 1998). The charge on PAs is distributed along the length of the carbon chain, conferring them distinct properties when compared to bivalent cations with point charges, like Mg^{2+} (Igarashi and Kashiwagi, 2000). The most functionally important PAs are putrescine (PUT), spermidine (SPD), and spermine (SPM). These molecules attract high interest because they affect multiple cellular processes, by engaging in covalent and noncovalent interactions with a wide variety of cellular targets, including proteins, nucleic acids, and phospholipids (Cohen, 1998). Consequently, PAs in cells occur not only as free molecules, but also as conjugates with small molecules, soluble in perchloric acid (PS-conjugated), or with macromolecules which are insoluble (PIS-conjugated) (Yang et al., 2010). Given that PAs affect many cellular processes, their intracellular pools are maintained within a relatively narrow range of concentrations, through the collective effects of an anabolic pathway and uptake mechanism on the one hand, and a catabolic pathway and an efflux mechanism on the other (Pegg, 2006, 2008; Casero and Pegg, 2009; Perez-Leal and Merali, 2012).

The natural PAs are formed from the decarboxylation products of ornithine and *S*-adenosyl-methionine in nearly all eukaryotic cells (Pegg, 2006). More recently, it has been demonstrated that arginine can be decarboxylated to agmatine, a diamine which inhibits proliferation by suppressing intracellular PA levels (Agostinelli et al., 2010). The efflux of PAs is facilitated after their acetylation by spermidine/spermine-N^1-acetyltransferase (SSAT), a key metabolic regulator (Pegg, 2008). Alternatively, the acetylated products can undergo decomposition catalyzed by acetylpolyamine oxidase (APAO), a process leading to cogeneration of H_2O_2 (Pegg, 2008).

Abbreviations: AAS, atomic absorption spectrophotometry; APAO, acetylpolyamine oxidase; LP, labilization period; MDA, malondialdehyde; MS, mass spectrometry; MT, metallothionein; ODC, ornithine decarboxylase; ROS, reactive oxygen species; PA, polyamine; PIS-conjugated PAs, perchloric acid-insoluble conjugated polyamines; PS-conjugated PAs, perchloric acid-soluble conjugated polyamines; PUT, putrescine; RP-HPLC, reverse phase high-performance liquid chromatography; SOD, superoxide dismutase; SPD, spermidine; SPM, spermine; SSAT, sperrmine/spermidine-N^1-acetyltransferase; TBARS, thiobarbituric reactive substances.

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Apart from the information that has been gathered about the PA effect on cell cycle regulation, gene expression, and signal transduction, an impressive amount of data has accumulated, pointing to the fundamental role of PAs in scavenging reactive oxygen species (ROS), generated by a number of biological systems expanding from prokaryotic organisms to human cells and organs (Matkovics et al., 1993; Farriol et al., 2003; Bellé et al., 2004; Tzirogiannis et al., 2004; Rider et al., 2007; Smirnova et al., 2012). Similar studies have also been conducted in chemical or in vitro enzymatic reactions directly producing free radicals (Drolet et al., 1986; Das and Misra, 2004). In both cases, the efficacy of the scavenging effect appears to correlate with the extent of PA amination, thus suggesting the involvement of amino groups in the mechanism of action (Das and Misra, 2004; Rider et al., 2007). Due to its four amino groups, SPM appears to be the most effective of the naturally occurring PAs in hydroxyl radical scavenging and singlet oxygen quenching (Ha et al., 1998).

In plants, overproduction of PAs constitutes an important component of the defense mechanisms against various environmental stresses, such as metal-mediated stress (Groppa et al., 2007; Yang et al., 2010; Serrano-Martinez and Casas, 2011; Xu et al., 2011) and salt-mediated stress (Groppa and Benavides, 2008). However, relative studies in marine organisms are scarce (Lovett and Watts, 1995; Yang et al., 2010; Lockwood and Somero, 2011), albeit many aquatic organisms have been identified as efficient accumulators of metals. For instance, mussels, such as *Mytilus galloprovincialis* are capable of accumulating trace metals and producing specific phenotypes. To the best of our knowledge, phenotypic plasticity in mussels exposed to metals has been traced so far to lipid and nucleic acid metabolisms, signal transduction pathways, and alterations in protein synthesis (Franzellitti and Fabbri, 2005; Fraser and Rogers, 2007; Koukouzika and Dimitriadis, 2008; Pytharopoulou et al., 2011; Vouras and Dailianis, 2012; Fokina et al., 2013), but not to changes in PA metabolism. Most studies measured free PAs in unstressed marine bivalves (Zappia et al., 1978; Hamana et al., 1991; Gasparini and Audit, 2000), therefore little is known about the potential implication of polyamine metabolism toward stressful conditions and the correlation between PA levels and biomarkers of oxidative stress and antioxidant defense.

Mussels are used as the bioindicators of choice in biomonitoring programs. At the same time, they occupy a key position in the trophic chain and are of great commercial value. On the other hand, Cd is a non-essential trace element, without any known physiological function. Although it does not undergo redox cycling, by entering the cells it promotes significant production of superoxide and hydroxyl radicals and severe lipid peroxidation, a fact suggesting unbalanced ROS generation (Cuypers et al., 2010; Pytharopoulou et al., 2011). Hence, Cd^{2+}-contaminations have attracted public attention worldwide. In the present work, we investigate the effect of accumulated bivalent cadmium (Cd^{2+}) in digestive glands of mussels *M. galloprovincialis* on the intracellular PA pools. In parallel, standard biomarkers of oxidative stress are analyzed to allow correlation with changes in PA pools. Our results shed light on the response of PA metabolism in mussels to Cd^{2+}-mediated stress, and suggest that induction of PA synthesis may have a potential implication in alleviating Cd^{2+}-induced stress in *M. galloprovincialis*, an effect possibly capable to be exploited as a suitable biomarker of Cd^{2+}-pollution.

2. Materials and methods

2.1. Exposure of mussels to Cd^{2+}

Mussels (*M. galloprovincialis*) of similar body size (6.0 ± 0.3 cm shell length), obtained from a marine farm (Poseidon Co.,Mandros; Galaxidi, Southern Greece), were kept unfed in tanks containing natural seawater, constantly aerated, to allow acclimation at 18 °C for 1 week, under natural photoperiod. During the experimentation, the water was changed every two days, while 38 mg of food (PROCORAL PHYTON, Tropic Marin, Wartenberg, Germany) were added in each tank, daily, in two doses. Thereafter, acute and subchronic Cd^{2+} exposures were performed. The acute treatment was conducted using groups of 40 individuals per tank (40 L) exposed to 100 μg/L Cd^{2+}, for 5 days. Longer exposures for 10 and 15 days were also carried out to provide a comparison with the subchronic exposures. The exposure concentration chosen, albeit high, has been reported by several field and laboratory studies (Bolognesi et al., 1999; Geret and Cosson, 2002; Ebrahimi and Taherianfard, 2010; Chandurvelan et al., 2013; Luo et al., 2013). Neither mortality nor milder clinical signs were observed during the first 10 days of exposure, either in exposed or in control groups of mussels. Mortality of less than 5% was recorded only on the 15th day of exposure to 100 μg/L Cd^{2+}. The subchronic treatment was conducted using mussels exposed to 25 μg/L Cd^{2+} for up to 15 days, as this concentration is known to cause sublethal effects without causing mortality to *M. galloprovincialis* (Pytharopoulou et al., 2011). At the end of each exposure period, mussels were removed and used either directly for

Table 1
Effects of exposure to sublethal Cd^{2+} concentrations on metal bioaccumulation and oxidative stress/antioxidant defense parameters in digestive glands of *M. galloprovincialis*[a].

Parameter	Cd^{2+} concentration (μg/L)	Time of exposure (days)			
		0	5	10	15
Cd^{2+} in digestive glands (μg/g tissue dry mass)	control	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
	25	0.8 ± 0.1	30.6 ± 2.7[b]	45.6 ± 3.1[b]	74.4 ± 4.9[b]
	100	0.8 ± 0.1	120.0 ± 7.0[b]	205.0 ± 14.0[b]	230.0 ± 15.9[b]
SR (pmol/mg tissue protein)	control	2.2 ± 0.2	2.0 ± 0.2	2.1 ± 0.2	2.1 ± 0.2
	25	2.0 ± 0.2	4.6 ± 0.4[b]	5.5 ± 0.4[b]	8.0 ± 0.5
	100	2.2 ± 0.2	6.8 ± 0.5[b]	10.8 ± 0.9[b]	20.2 ± 1.0[b]
MDA (nmol/mg tissue protein)	control	1.8 ± 0.2	1.8 ± 0.2	1.9 ± 0.2	1.9 ± 0.2
	25	1.9 ± 0.2	2.4 ± 0.2[b]	3.2 ± 0.4[b]	4.0 ± 0.5[b]
	100	1.8 ± 0.2	2.7 ± 0.2[b]	5.0 ± 0.4[b]	5.6 ± 0.5[b]
LP (min)	control	29.0 ± 2.7	29.0 ± 2.2	27.0 ± 2.7	28.0 ± 2.7
	25	28.0 ± 2.2	18.0 ± 2.7[b]	16.2 ± 1.6[b]	15.2 ± 0.4[b]
	100	28.0 ± 2.7	10.0 ± 0.7[b]	7.5 ± 0.5[b]	6.2 ± 0.3[b]
SOD (units/mg tissue protein)	control	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
	25	0.7 ± 0.1	1.9 ± 0.1[b]	2.0 ± 0.1[b]	1.2 ± 0.1[b]
	100	0.7 ± 0.1	1.4 ± 0.1[b]	1.2 ± 0.1[b]	0.7 ± 0.1
MTs (μg/g tissue wet mass)	control	45.0 ± 3.3	42.0 ± 5.2	40.0 ± 4.0	55.0 ± 4.9
	25	40.0 ± 4.0	107.0 ± 9.7[b]	130.0 ± 11.9[b]	232.0 ± 14.2[b]
	100	40.0 ± 4.0	170.0 ± 20.0[b]	270.0 ± 19.0[b]	342.0 ± 28.0[b]

Abbreviations: LP, labilization period; SR, superoxide radical; SOD, superoxide dismutase; MDA, malondialdehyde; and MTs, metallothioneins.
[a] Each value is given as mean ± S.D. (n = 5).
[b] Significantly different values from those measured in control samples ($p < 0.05$).

the superoxide radical assay or their digestive glands were dissected and prepared for further analysis.

2.2. Metal analysis

Cadmium was analyzed in seawater after each change of water in tanks to correct any alterations in metal concentration due to the metal uptake by mussels, by flameless atomic absorption spectrophotometry (AAS), according to Jan and Young (1978). Cadmium in pooled digestive glands, excised from 5 mussel specimens, was also determined by AAS in dried samples (80 °C), after digestion with nitric acid (Stien et al., 1998). Merck standard solutions and certified reference materials (IAEA-436 tuna fish flesh homogenate) were used for calibration and verification of the accuracy of the analysis. Metal concentration in seawater was expressed as μg/L, while in digestive glands as μg/g tissue (dry mass).

2.3. Biomarker analysis

Metallothionein (MT) content in digestive glands was evaluated according to a method described by Viarengo et al. (1997). The data were expressed in μg/g tissue (wet mass), assuming 21 cysteine residues per MT molecule and a molecular mass for MT in *M. galloprovincialis* equal to 8600 Da.

Superoxide radical was measured in situ according to Georgiou et al. (2005). Total proteins were determined in the tissue homogenate according to Bradford (1976), using bovine serum albumin as standard. Superoxide radical concentration was expressed in pmol/mg tissue protein.

Lipid peroxidation was determined by quantifying the amount of thiobarbituric reactive substances (TBARS) in digestive glands, as described by Zamora et al. (1997). TBARS concentrations were calculated from an external standard curve of malondialdehyde (MDA) and the values were expressed as nmol of MDA equivalents formed per mg tissue protein.

Lysosomal membrane stability in 10-μm thick cryostat sections of mussel digestive glands was determined by measuring the lysosomal permeability in AS-BI *N*-acetyl-β-D-glucosaminidine, an exogenously added substrate of the lysosomal *N*-acetyl-β-hexosaminidase (Moore, 1985). The permeability was assessed by measuring the labilization period (LP), which is the preincubation time that is required to completely labilize the lysosomal membranes, at acid conditions and 37 °C.

Superoxide dismutase (SOD; EC 1.15.1.1) activity in digestive glands was determined by monitoring the inhibitory activity of SOD on the reduction of oxidized dianisidine by superoxide radical, as described previously (Pytharopoulou et al., 2011). One unit of SOD was defined as the amount of SOD that inhibits the rate of dianisidine reduction by 50%.

2.4. Polyamine analysis

Polyamine levels in digestive gland were determined by reverse phase high-performance liquid chromatography (RP-HPLC-UV). For these assays, 100 mg tissue (wet mass) were homogenized in 1 mL 0.6 M $HClO_4$, incubated in ice for 1 h, and centrifuged at 12,000 ×*g* for 20 min at 4 °C. The pellet was extracted three times with 1 mL 0.6 M $HClO_4$ and recentrifuged. The four supernatants were pooled and used to determine the levels of free PAs. For quantification of PS-conjugated PAs, 2 mL of the supernatant was mixed with 2 mL 12 N HCl, and hydrolyzed for 18 h at 110 °C in tightly capped test tubes. The hydrolysate was filtered, dried, and then resuspended in 2 mL 0.6 M $HClO_4$. Analysis of PAs in this hydrolyzed fraction gives the sum of free PAs and PS-conjugated PAs. For quantification of PIS-conjugated PAs, the pellet was rinsed twice with 2 mL 0.6 M $HClO_4$ to remove any trace of soluble PAs, and then dissolved in 2 mL 1 N NaOH. The mixture was centrifuged at 12,000 ×*g* for 20 min and the supernatant was hydrolyzed with 12 N HCl as above. Each sample, supernatant, hydrolyzed supernatant, or hydrolyzed pellet, after dansylation and extraction of the dansyl derivatives by toluene (Outinen et al., 1995), was dried, redissolved in 10 mM phosphate buffer pH 4.4 and fractionated by RP-HPLC (Waters 600 HPLC system equipped with a tunable UV absorbance detector Waters 486), using a C_{18}-Symmetry, 3.5-μm, 75 mm × 4.6 mm column obtained from Waters. For the separation of free PAs and acetylated PAs, gradient I and gradient II were used, respectively, designed and optimized by Kabra et al. (1986). The flow rate was set at 1 mL/min and the separation was performed at 25 °C. PA species were identified by comparing retention times with commercial PA and acetyl-PA standards (Sigma). 1,6-diaminohexane was used as internal standard. To verify the identification of peaks, fractions eluted under each peak area were pooled, cleared of phosphate ions, dried, solved in CH_3OH and analyzed by a quadrupole mass spectrometer (Water Micromass ZQ), equipped with Masslynx 4.1 software. Electron-spray ionization (ESI, ES+) mass spectra were recorded at 30 V. Polyamine peaks were monitored by

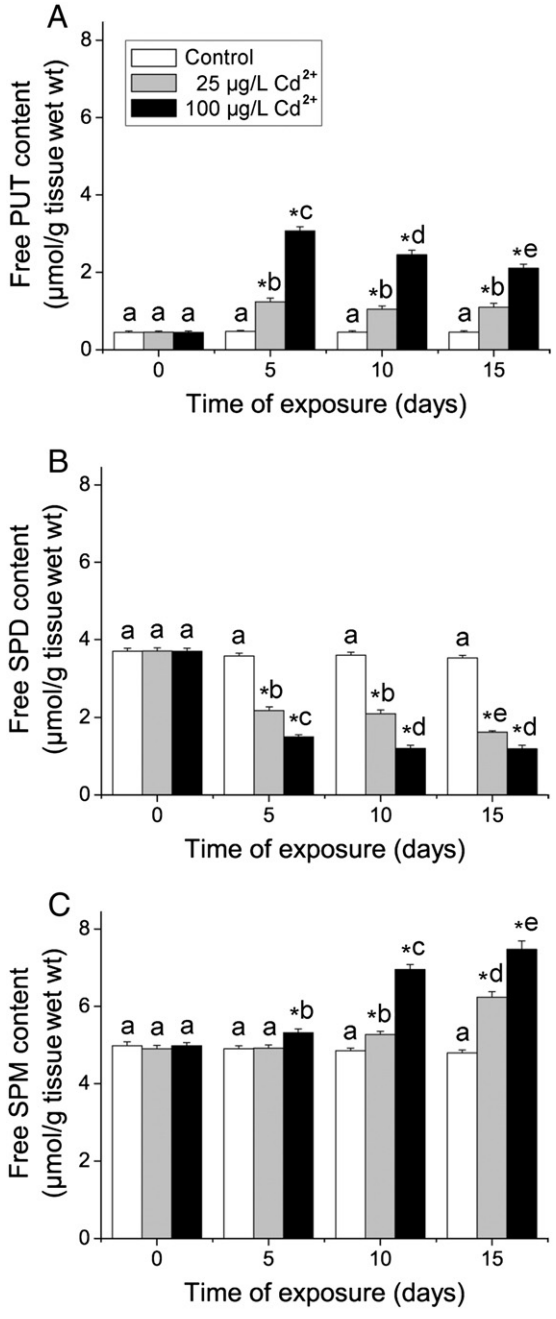

Fig. 1. Effect of mussel exposure to Cd^{2+} on the content of free PAs in digestive glands. Abbreviations: PAs, polyamines; PUT, putrescine; SPD, spermidine and SPM, spermine.

UV absorption at 258 nm, using the flow-through spectrophotometer cell incorporated in the RP-HPLC unit. Calibration curves were obtained, using authentic standards analyzed with the same method used for cell samples. A linear relationship between peak area and concentration was observed for all PAs and acetylated PAs, with a regression coefficient (r^2), better than 0.985. The detection limits for PUT, acetyl-PUT, SPD, acetyl-SPD, SPM, and acetyl-SPM were 52 pmol, 33 pmol, 18 pmol, 57 pmol, 36 pmol, and 2 pmol, respectively. The reproducibility (C.V.%) of the intra-assays ranged from 3 to 5, while those of the inter-assays ranged from 5 to 9. The average recoveries of PAs determined by adding standards at three levels ranged from 97% to 102%. Data processing was made, using the Millenium 32 software-Waters, and the results were expressed as μmol PA or acetylated PA/g tissue (wet mass).

2.5. Statistical analysis

All measurements were obtained from at least five samples, with two replicates per sample, and the data were expressed as means ± standard deviation. Significant differences between mean values were measured by the F-Scheffé test, while correlations among variables were calculated using the two-tailed Pearson correlation method (SPSS program 20.0 for Windows). Values designated in Tables and Figures with different letters are significantly different at $p < 0.05$, while asterisks mark statistically significant difference from the control value.

3. Results and discussion

3.1. Characterization of oxidative stress induced by Cd^{2+}

Cadmium concentrations remained very low in the digestive glands of mussels exposed to clean seawater (control mussels), throughout the exposure period. In exposed mussels to 25 μg/L Cd^{2+} and 100 μg/L Cd^{2+}, accumulation of the metal in digestive glands was found to increase in a concentration- and time-dependent manner, reaching a value of 74.4 and 230 μg/g tissue (dry mass), respectively, at the 15th day of exposure (Table 1). This is in agreement with previous studies performed by our group and others, which indicated a low capability of mussels of eliminating this metal (Viarengo et al., 1985; Pytharopoulou et al., 2011, 2013). At the 5th day of exposure, the accumulated metal caused a concentration-dependent increase in superoxide radical production and lipid peroxidation (Tables 1, 3), a fact indicating the beginning of a series of oxidative stress events. Consistently, exposure of mussels to 25 μg/L Cd^{2+} and 100 μg/L Cd^{2+} for 5 days caused 38% and 65.5% reductions, respectively, in lysosomal membrane stability (Table 1). It is known that lysosomes in the digestive cells of mussels constitute the main intracellular sites of Cd^{2+} accumulation (Regoli, 1992); in turn, Cd^{2+} accumulation in lysosomes causes alterations in lysosomal membrane integrity (Domouhtsidou et al., 2004). In parallel, SOD, the enzyme that dismutases superoxide radical into H_2O_2, and MTs underwent significant increases (Table 1), both considered as the most prominent members of the antioxidant defense.

Following prolonged exposure periods to Cd^{2+}, the increase in superoxide radical production, lipid peroxidation, lysosomal membrane instability, and MT content became more evident. In contrast, SOD activity progressively turned back to the control levels (Table 1). As will be shown below (Table 3), this abnormal behavior leads to a lack of correlations between SOD activity and Cd^{2+} concentration or content of PAs. Possible explanations about this phenomenon might be that Cd^{2+} at high concentrations could displace metal cofactors in the catalytic center of SOD, or probably unbalanced production of H_2O_2 could suppress SOD activity by a product-inhibition mechanism, as previously hypothesized (Viarengo et al., 1985; Wang and Wang, 2009; Pytharopoulou et al., 2011). The finding that exposure of mussels to 25 μg/L Cd^{2+} causes stronger induction of SOD than that recorded after exposure to 100 μg/L Cd^{2+}, strengthens these hypotheses.

Our data fit well with previous studies published by our group (Pytharopoulou et al., 2011, 2013) and others (Geret and Cosson, 2002; Geret et al., 2002; Chandurvelan et al., 2013). In a recent study, Koutsogiannaki et al. (2014) reported that treatment of mussel hemocytes with 5 μM (= 562 μg/L) Cd^{2+} for 30 min triggers 35% and 30% increases in superoxide radical production and lipid peroxidation, respectively. The relatively higher changes in both biomarkers observed in our study at the 15th day of exposure (over than 3.8-fold and 2.1-fold increases compared to controls, respectively), appear to be due to the longer time of exposure, the higher tissue Cd^{2+} concentration, and probably to a different digestive gland capability of responding to Cd^{2+}.

3.2. Effects of Cd^{2+} on free PA content

The main polyamine species measured in the digestive glands of untreated mussels were PUT, SPD and SPM, with the latter polyamine being in larger amounts (Fig. 1). Sym-nor-SPD (1,7-diamino-4-azaheptane) was found at very small amounts (250 nmol/g tissue wet mass) not altered during the exposure of mussels to Cd^{2+}. Although the proportion of polyamine species measured in the present work resembles of that previously detected in whole homogenates of *M. galloprovincialis* (Zappia et al., 1978) and *Grassostrea gigas* (Gasparini and Audit, 2000), the concentration of each free polyamine in digestive gland was relatively higher, a sign that this tissue represents the main producer of polyamines, as suggested by others (Asotra et al., 1988; Orlandini et al., 1989). Compared to controls, mussels exposed for 5 days to 25 μg/L Cd^{2+} showed a 2.6-fold enhancement of PUT content in their digestive glands, 40% reduction in SPD content, and no

Table 2
Sum of free PAs (PUT + SPD + SPM), sum of total PAs, and the ratio (SPD + SPM)/PUT in extracts of digestive glands from *M. galloprovincialis* exposed to Cd^{2+} [a].

Parameter	Concentration (μg/L)	Time of exposure (days)			
		0	5	10	15
$PUT_{free} + SPD_{free} + SPM_{free}$ (μmol/g tissue wet mass)	control	9.1 ± 0.5	8.9 ± 0.4	8.9 ± 0.4	8.8 ± 0.4
	25	9.1 ± 0.4	8.3 ± 0.4	8.4 ± 0.5	8.9 ± 0.5
	100	9.2 ± 0.5	9.9 ± 0.5	10.6 ± 0.6[b]	10.8 ± 0.5[b]
$(SPD_{free} + SPM_{free})/PUT_{free}$	control	19.4 ± 1.5	18.0 ± 1.2	18.8 ± 1.2	18.5 ± 1.3
	25	19.1 ± 1.3	5.7 ± 0.5[b]	7.0 ± 0.7[b]	7.2 ± 0.7[b]
	100	18.2 ± 1.5	2.2 ± 0.2[b]	3.3 ± 0.3[b]	4.1 ± 0.3[b]
Total PAs (μmol/g tissue wet mass)	control	9.4 ± 0.5	9.2 ± 0.4	9.3 ± 0.4	9.2 ± 0.4
	25	9.4 ± 0.5	9.3 ± 0.6	10.5 ± 0.5[b]	12.4 ± 0.6[b]
	100	9.6 ± 0.5	12.4 ± 0.5[b]	15.7 ± 0.7[b]	19.4 ± 0.6
$(SPD_{total} + SPM_{total})/PUT_{total}$	control	15.9 ± 1.5	15.0 ± 1.3	15.3 ± 1.4	15.0 ± 1.4
	25	15.7 ± 1.5	5.3 ± 0.5[b]	5.8 ± 0.5[b]	5.9 ± 0.5[b]
	100	16.0 ± 1.5	2.5 ± 0.2[b]	3.7 ± 0.3[b]	4.9 ± 0.3[b]

[a] Each value is given as mean ± S.D. (n = 5).
[b] Significantly different values from those measured in control samples ($p < 0.05$).

alterations in SPM content. Exposure to 100 μg/L Cd^{2+} caused, respectively, a 7-fold enhancement of PUT content, 60% reduction in SPD content, and a modest increase in SPM content (Fig. 1). This is in agreement with a wealth of data in literature, indicating that many types of environmental stress can stimulate the activity of ornithine decarboxylase (ODC), an enzyme that catalyzes the decarboxylation of ornithine to PUT (Pegg, 2006; Lockwood and Somero, 2011; Perez-Leal and Merali, 2012). Noteworthy, ODC contains in its promoter many elements responding to several transcription factors (Bachrach et al., 2001), and it is considered as the rate-limiting enzyme of the polyamine biosynthesis (Casero and Pegg, 2009).

Following prolonged exposure periods to Cd^{2+}, PUT gradually declined to reach increments of 2.4- and 4.7-folds at the 15th day of exposure to 25 μg/L and 100 μg/L Cd^{2+}, respectively (Fig. 1). Meanwhile, SPD levels further decreased, whereas SPM levels increased. At a first glance, it appears that Cd^{2+}-mediated stress inhibits the conversion of PUT to SPD, whereas accelerating the synthesis of SPM from SPD.

To better display the impact of Cd^{2+} on free PAs, we calculated the sum of free PAs (PUT + SPD + SPM), as well as the ratio (SPD + SPM)/PUT throughout the exposure period to Cd^{2+}. As shown in Table 2, exposure of mussels to 25 μg/L and 100 μg/L Cd^{2+} for 5 days marginally influenced the sum of free PAs, however it threw the value of the ratio from 18.0 to 5.7 and 2.2, respectively. Following prolonged exposure periods to Cd^{2+}, the sum of free PAs remained almost constant, whereas the value of the ratio progressively elevated. Taken together, these results imply that mussels respond to the Cd^{2+}-mediated stress by stimulating the PUT and SPM biosynthesis. At the early phase of exposure, when oxidative stress is quite unbalanced, the abrupt increase in PUT levels results in a sharp drop of (SPD + SPM)/PUT ratio. By exposing the mussels to Cd^{2+} for prolonged time, the value of ratio is elevated as a result of the generation of SPM. Given that SPM exhibits superior activity in scavenging superoxide radicals, quenching singlet oxygen, and chelating metal ions (Ha et al., 1998), generation of SPM seems to be important in improving tolerance of mussels against Cd^{2+}-mediated stress.

3.3. Effects of Cd^{2+} on PS-conjugated PAs

PAs are capable of binding to small molecules, remaining at the supernatants of perchloric acid extracts. Many studies have detected such conjugates in cellular extracts of plant or animal tissues exposed to abiotic and biotic stresses, attributing them an important role in defense mechanisms (Alcázar et al., 2006; Wimalasekera et al., 2011). In accordance, we observed that mussels exposed to 100 μg/L Cd^{2+} for 5 days contained higher levels of PS-conjugated PUT, SPD, and SPM in their digestive glands, when compared to control mussels (Fig. 2). At the 15th day of exposure to 100 μg/L Cd^{2+}, the increase in PS-conjugated PA content was much higher, in particular those of PS-conjugated SPM (46-fold rise; Fig. 2). Exposure of mussels to 25 μg/L Cd^{2+} resulted in a similar trend of increments, but clearly of less intensity (Fig. 2). It should be mentioned that PS-conjugated PAs are the most over-amplified PA species under exposure to Cd^{2+}, a fact suggesting that the defense mechanisms of mussels against Cd^{2+}-mediated stress require the involvement of PS-conjugated PAs than free PAs.

3.4. Effects of Cd^{2+} on PIS-conjugated PAs

PIS-conjugated PAs represent the polyamine fraction, in which PAs are associated with macromolecules, like proteins, nucleic acids, and lipids. We found that PIS-conjugated PA levels, in particular those of SPD and SPM, increase as the accumulation of Cd^{2+} in digestive glands elevates (Fig. 3). As suggested by other studies (Dondini et al., 2001; Serrano-Martinez and Casas, 2011; Xu et al., 2011), SPD and SPM, due to their higher content in amino groups per molecule compared to those of PUT, exhibit higher affinity towards macromolecules and, therefore, operate as better stabilizers of their structure under Cd^{2+}-mediated stress.

3.5. Acetylation of PAs is influenced by Cd^{2+}-mediated stress

To obtain a complete picture of PA metabolism under Cd^{2+}-mediated stress, the levels of acetyl-PAs were also measured. Acetylation is a means to remove positive charges from PAs and alter their binding properties and transport through lipid bilayers. A predominant role in acetylation of cytosolic SPD and SPM is played by SSAT, a key metabolic regulator of PA catabolism and homeostasis (Pegg, 2008).

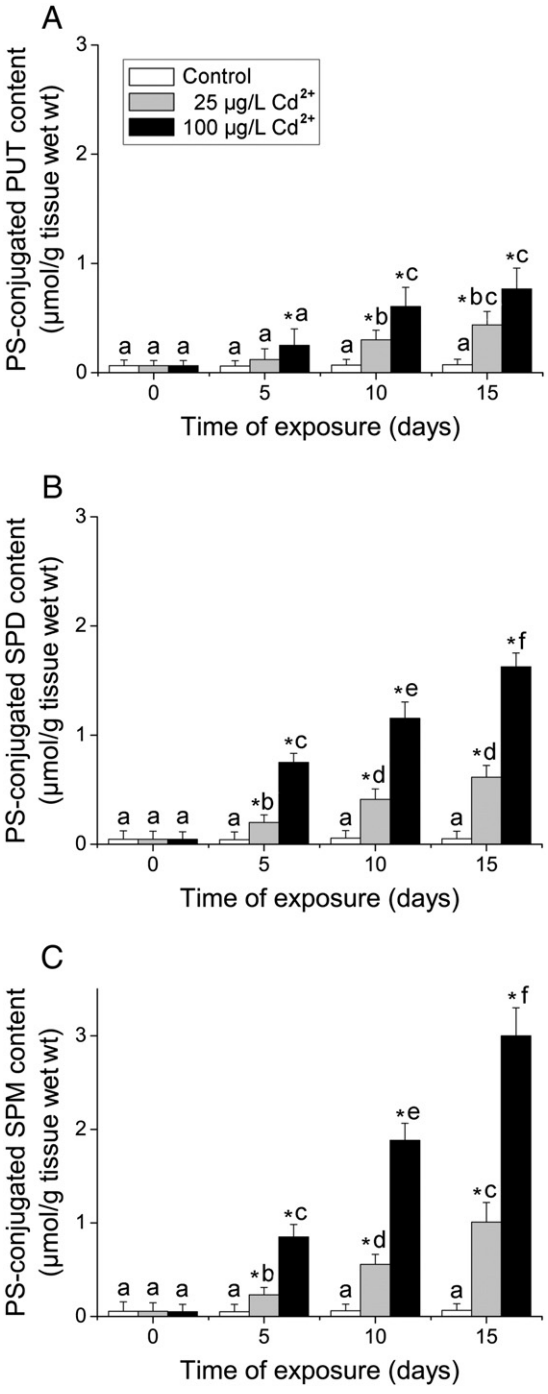

Fig. 2. Effect of mussel exposure to Cd^{2+} on the content of PS-conjugated PAs in digestive glands. Abbreviations: PAs, polyamines; PS, soluble in perchloric acid; PUT, putrescine; SPD, spermidine and SPM, spermine.

The acetylated products of the reaction are good substrates for APAO, an enzyme that subsequently cleaves the acetyl-PAs to generate *N*-acetylamino-propanal, PAs of smaller size, and H_2O_2, thus effectively reversing the anabolic reactions. Evidence provided by several studies in terrestrial and marine organisms suggests that SSAT, and at a lesser degree APAO, are activated by oxidative stress (Casero and Pegg, 2009; Yang et al., 2010; Smirnova et al., 2012). Data of our study supporting this suggestion are related only with changes in acetyl-SPD. As shown in Fig. 4, the levels of acetyl-SPD increased concomitantly with the amount of the accumulated Cd^{2+} in digestive glands. In contrast, Cd^{2+}-mediated stress exerted on acetyl-SPM level an opposite effect. Acetyl-PUT concentration, after a burst at the 5th day of exposure, followed a similar descent trend. The burst of acetyl-PUT at the early phase of exposure to Cd^{2+} cannot be attributed to SSAT activation, since PUT is not used by SSAT as a substrate. Nevertheless, acetylation of PUT can be catalyzed by other nuclear or cytosolic acetyltransferases acting when PUT is in abundance (Seiler, 1987).

3.6. An overview of mussel PA metabolism under Cd^{2+}-mediated stress

Combining the results presented in Figs. 1, 2, and 3, we got at depicting the evolution of the sum of the levels of active PAs (free, PS-conjugated, PIS-conjugated). As shown in Table 2, the sum of PAs (total PAs) was increased linearly with the time of exposure to Cd^{2+} ($R = 0.999$, $p < 0.01$). Moreover, Cd^{2+}-stress disturbed the relative predominance of each PA species. Namely, the concentration of total PUT underwent a huge increase at the 5th day of exposure and then slightly decreased, remaining however higher than in controls. This profile of changes could be attributed to an induction of ODC activity or to a

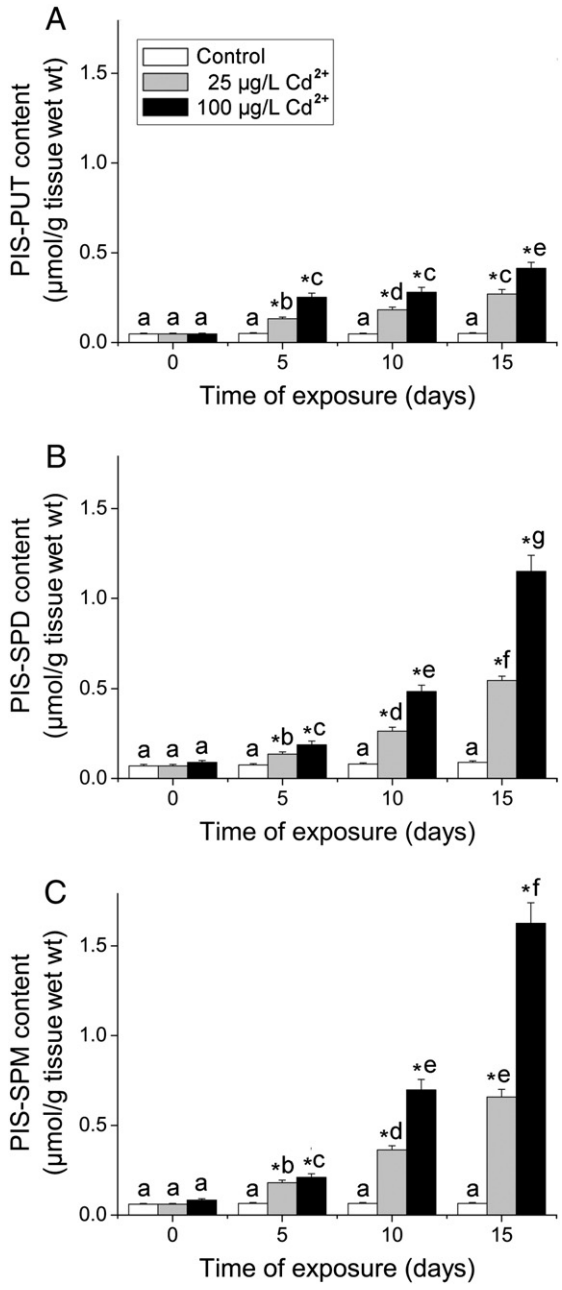

Fig. 3. Effect of mussel exposure to Cd^{2+} on the content of PIS-conjugated PAs in digestive glands. Abbreviations: PAs, polyamines; PIS, insoluble in perchloric acid; PUT, putrescine; SPD, spermidine and SPM, spermine.

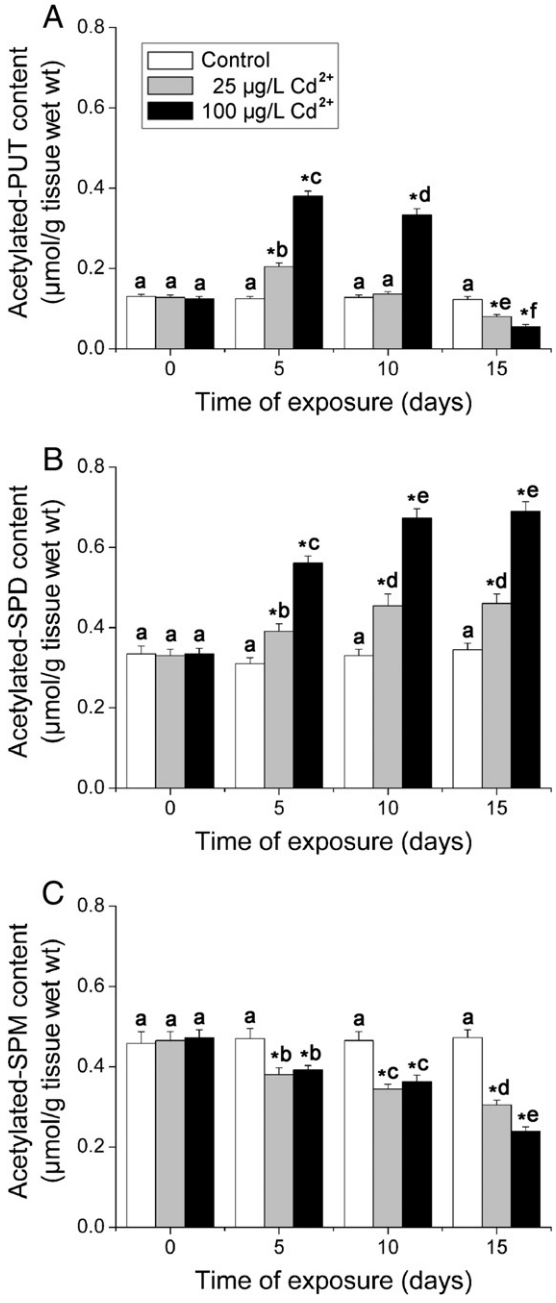

Fig. 4. Effect of mussel exposure to Cd^{2+} on the content of acetylated PAs in digestive glands. Acetyl-SPD content denotes the sum of N^1-acetyl-SPD and N^8-acetyl-SPD concentrations. Abbreviations: PAs, polyamines; PUT, putrescine; SPD, spermidine and SPM, spermine.

conversion of SPM and SPD to PUT, catalyzed by the concerted action of SSAT and APAO. As acetylated polyamines were generally kept at low levels throughout the exposure period, the first hypothesis seems more likely. Nonetheless, accurate contribution of each of the two pathways cannot be easily evaluated, without concomitant assessment of the involved enzymes in each pathway. The content of total SPM continuously increased during the exposure, either to 25 μg/L or to 100 μg/L Cd^{2+}, while total SPD content declined at the 5th day of exposure and then gradually returned to the control value. As a consequence, the ratio $(SPD_{total} + SPM_{total})/PUT_{toatal}$ underwent a sharp decrease at the early phase of exposure to Cd^{2+}, and then started to increase, reaching an intermediate value at the end of exposure (Table 2). This profile of changes looks very similar to that seen in free PAs.

Correlation analysis between oxidative-stress or antioxidant-defense parameters (Table 1) and free or conjugated PAs, is given in Table 3. The main conclusions drawn from Table 3 can be summarized as follows: in acute, and more emphasized in subchronic treatments of mussels with Cd^{2+}, there was a negative correlation between free PUT or free SPM levels and LP values, as well as a significant positive correlation with superoxide production, lipid peroxidation, MT concentration, and cytosolic Cd content. However, no correlation was found between free PUT or SPM levels and SOD activity. Compared with PUT or SPM, free SPD levels displayed significant but opposite correlations with the tested biomarkers. A similar pattern of correlations was observed for PS- or PIS-conjugated PAs, except for one case; PS- or PIS-conjugated SPD, compared with free SPD, displayed opposite correlations with the oxidative-stress biomarkers. Noteworthy, a strong correlation was recorded between the signals of oxidative stress and the ratio (SPD + SPM)/PUT, expressed in terms either of free or total PAs.

It is evident from Table 3 that there is a significant correlation between PA levels and biomarkers of Cd^{2+}-mediated toxicity. Recently, Fokina et al. (2013) suggested the use of lipid and fatty acids composition parameters as biomarkers reflecting the adverse effects of cadmium and copper on bivalve mollusks. Besides the contradictory views concerning the use of lipids as biomarkers of Cd-pollution (Koukouzika and Dimitriadis, 2008), the magnitude of changes in lipid and fatty acid composition observed by Fokina et al. is quite moderate, even after exposure of mollusks to 500 μg/L Cd^{2+} for 3 days, a fact limiting the use of such biomarkers in elucidating toxic mechanisms. In contrast, we observed highly discernible changes in the levels of certain PAs upon exposure to Cd^{2+} (for instance, see Figs. 2 and 3). Whether these changes also hold specificity features, in terms of differentiating among trace metals, deserves further investigation.

4. Conclusions

Accumulation of Cd^{2+} in mussel digestive glands results in the establishment of oxidative stress, characterized by elevated production of superoxide radical, impairment of lysosomal membranes, and high levels of lipid peroxidation. The digestive gland cells respond to Cd^{2+}-mediated stress by the induction of superoxide dismutase activity, stimulation of metallothionein biosynthesis, changes in PA metabolism, and possibly by other defense mechanisms not examined in this work. The profile of PA alterations is characterized by an overproduction of free PUT, which eventually leads to increased levels of free SPM, the most efficient PA in ROS scavenging. While the sum of free PAs does not significantly change during the 15 days exposure period, the sum of total PAs (free PAs + PS-conjugated PAs + PIS-conjugated PAs) upon exposure to 25 μg/L Cd^{2+} increases by 30%, while upon exposure to 100 μg/L Cd^{2+} it redoubles. This is due to a dramatic increase in the content of conjugated PAs, a fact emphasizing their role in the tolerance of cells against Cd^{2+}-mediated stress. By fitting the content of each PA on the ratio (SPD + SPM)/PUT, expressed in terms either of free or total PAs, we concluded that it could be a good indicator of stress caused by Cd^{2+}. However, to gain a more profound understanding of the interplay in mussels between changes of the PA pattern and Cd^{2+}-mediated stress, future studies should focus on the expression of enzymes participating in the PA metabolism.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Table 3
Correlation coefficients between oxidative stress biomarkers and PA variables tested in the digestive gland of *M. galloprovincialis* exposed to Cd^{2+} for 5 or 15 days.

	LP	SR	SOD	MDA	MTs	Tissue Cd^{2+}
Exposure for 5 days						
PUT	−0.930 (<0.01)	0.941 (<0.01)	0.350 (0.20)	0.831 (<0.01)	0.959 (<0.01)	0.984 (<0.01)
SPD	0.884 (<0.01)	−0.958 (<0.01)	−0.700 (<0.01)	−0.867 (<0.01)	−0.937 (<0.01)	−0.867 (<0.01)
SPM	−0.456 (0.08)	0.447 (0.09)	−0.013 (0.96)	0.647 (<0.01)	0.577 (0.02)	0.497 (0.06)
PS-PUT	−0.712 (<0.01)	0.676 (<0.01)	0.242 (0.38)	0.523 (0.04)	0.679 (<0.01)	0.684 (<0.01)
PS-SPD	−0.940 (<0.01)	0.918 (<0.01)	0.271 (0.33)	0.777 (<0.01)	0.919 (<0.01)	0.977 (<0.01)
PS-SPM	−0.913 (<0.01)	0.916 (<0.01)	0.299 (0.28)	0.742 (<0.01)	0.899 (<0.01)	0.968 (<0.01)
PIS-PUT	−0.995 (<0.01)	0.961 (<0.01)	0.459 (0.08)	0.810 (<0.01)	0.957 (<0.01)	0.979 (<0.01)
PIS-SPD	−0.939 (<0.01)	0.952 (<0.01)	0.552 (0.03)	0.851 (<0.01)	0.946 (<0.01)	0.913 (<0.01)
PIS-SPM	−0.859 (<0.01)	0.915 (<0.01)	0.766 (<0.01)	0.841 (<0.01)	0.911 (<0.01)	0.813 (<0.01)
(SPD + SPM)/PUT	0.857 (<0.01)	−0.932 (<0.01)	−0.781 (<0.01)	−0.851 (<0.01)	−0.921 (<0.01)	−0.824 (<0.01)
Exposure for 15 days						
PUT	−0.956 (<0.01)	0.986 (<0.01)	−0.147 (0.60)	0.940 (<0.01)	0.947 (<0.01)	0.994 (<0.01)
SPD	0.898 (<0.01)	−0.838 (<0.01)	−0.316 (0.25)	−0.923 (<0.01)	−0.959 (<0.01)	−0.839 (<0.01)
SPM	−0.912 (<0.01)	0.920 (<0.01)	−0.026 (0.93)	0.878 (<0.01)	0.916 (<0.01)	0.919 (<0.01)
PS-PUT	−0.921 (<0.01)	0.907 (<0.01)	−0.929 (<0.01)	0.952 (<0.01)	0.918 (<0.01)	0.909 (<0.01)
PS-SPD	−0.956 (<0.01)	0.986 (<0.01)	−0.154 (0.58)	0.930 (<0.01)	0.946 (<0.01)	0.989 (<0.01)
PS-SPM	−0.951 (<0.01)	0.989 (<0.01)	−0.215 (0.44)	0.930 (<0.01)	0.930 (<0.01)	0.992 (<0.01)
PIS-PUT	−0.951 (<0.01)	0.942 (<0.01)	0.096 (0.73)	0.963 (<0.01)	0.991 (<0.01)	0.940 (<0.01)
PIS-SPD	−0.973 (<0.01)	0.986 (<0.01)	−0.095 (0.74)	0.947 (<0.01)	0.969 (<0.01)	0.988 (<0.01)
PIS-SPM	−0.969 (<0.01)	0.987 (<0.01)	−0.135 (0.63)	0.950 (<0.01)	0.949 (<0.01)	0.986 (<0.01)
(SPD + SPM)/PUT	0.915 (<0.01)	−0.874 (<0.01)	−0.241 (0.39)	−0.935 (<0.01)	−0.964 (<0.01)	−0.870 (<0.01)

The probability level is shown in parenthesis.
Abbreviations used: LP, labilization period; SO, superoxide radical; SOD, superoxide dismutase; MDA, malondialdehyde; MTs, metallothioneins; PUT, putrescine; SPD, spermidine; SPM, spermine; PS-, perchloric acid-soluble conjugated; and PIS-, perchloric acid-insoluble conjugated.

References

Agostinelli, E., Marques, M.P.M., Calheiros, R., Gil, F.P.S.C., Tempera, G., Viceconte, N., Battaglia, V., Grancara, S., Toninello, A., 2010. Polyamines: fundamental characters in chemistry and biology. Amino Acids 38, 393–403.

Alcázar, R., Marco, F., Cuevas, J.C., Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A.F., Altabella, T., 2006. Involvement of polyamines in plant response to abiotic stress. Biotechnol. Lett. 28, 1867–1876.

Asotra, S., Mladenov, P.V., Burke, R.D., 1988. Polyamines and cell proliferation in the sea star *Pycnopodia helianthoides*. Comp. Biochem. Physiol. B 90, 885–890.

Bachrach, U., Wang, Y.C., Tabib, A., 2001. Polyamines: new cues in cellular signal transduction. News Physiol. Sci. 16, 106–109.

Bellé, N.A., Dalmolin, G.D., Fonini, G., Rubin, M.A., Rocha, J.B., 2004. Polyamines reduce lipid peroxidation induced by different pro-oxidant agents. Brain Res. 1008, 245–251.

Bolognesi, C., Landini, E., Roggieri, P., Fabbri, R., Viarengo, A., 1999. Genotoxicity biomarkers in the assessment of heavy metal effects in mussels: experimental studies. Environ. Mol. Mutagen. 33, 287–292.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Casero, R.A., Pegg, A.E., 2009. Polyamine catabolism and disease. Biochem. J. 421, 323–338.

Chandurvelan, B., Marsden, I.D., Gaw, S., Glover, C.N., 2013. Biochemical biomarker responses of green-lipped mussel, *Perna canaliculus*, to acute and subchronic waterborn cadmium toxicity. Aquat. Toxicol. 140–141, 303–313.

Cohen, S.S., 1998. A Guide to Polyamines. Oxford University Press, New York.

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A.R., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J., Smeets, K., 2010. Cadmium stress: an oxidative challenge. Biometals 23, 927–940.

Das, K.C., Misra, H.P., 2004. Hydroxyl radical scavenging and singlet oxygen quenching properties of polyamines. Mol. Cell. Biochem. 262, 127–133.

Domouhtsidou, G.P., Dailianis, S., Kaloyianni, M., Dimitriadis, V.K., 2004. Lysosomal membrane stability and metallothionein content in *Mytilus galloprovincialis* (L.) as biomarkers. Combination with trace metal concentrations. Mar. Pollut. Bull. 48, 572–586.

Dondini, L., Bonazzi, S., Del Duka, S., Bregoli, A.M., Serafini-Fracassini, D., 2001. Acclimation of chloroplast transglutaminase to high NaCl concentration in a polyamine-deficient variant strain in *Dunaliella salina* and its wild type. J. Plant Physiol. 158, 185–197.

Drolet, G., Dumbroff, E.B., Legge, R.L., Thompson, J.E., 1986. Radical scavenging properties of polyamines. Phytochemistry 25, 367–371.

Ebrahimi, M., Taherianfard, M., 2010. Concentrations of four heavy metals (cadmium, lead, mercury, and arsenic) in organs of two cyprinid fish (*Cyprinus carpio* and *Capoeta* sp.) from the Kor River (Iran). Environ. Monit. Assess. 168, 575–585.

Farriol, M., Segovia-Silvestre, T., Venereo, Y., Orta, X., 2003. Antioxidant effect of polyamines on erythrocyte cell membrane lipoperoxidation after free-radical damage. Phytother. Res. 17, 44–47.

Fokina, N.N., Ruokolainen, T.R., Nemova, N.N., Bakhmet, I.N., 2013. Changes of blue mussels *Mytilus edulis* L. lipid composition under cadmium and copper toxic effect. Biol. Trace Elem. Res. 154, 217–225.

Franzellitti, S., Fabbri, E., 2005. Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. Biochem. Biophys. Res. Commun. 336, 1157–1163.

Fraser, K.P.P., Rogers, A.D., 2007. Protein metabolism in marine animals: the underlying mechanism of growth. Adv. Mar. Biol. 52, 267–362.

Gasparini, S., Audit, C., 2000. The free guanidine and polyamine pools of bivalve mollusks in relation to their ecology. Biochem. Syst. Ecol. 28, 209–218.

Georgiou, C.D., Papapostolou, I., Patsoukis, N., Tsegenidis, T., Sideris, T., 2005. An ultrasensitive fluorescent assay for the in vivo quantification of superoxide radical in organisms. Anal. Biochem. 347, 144–151.

Geret, F., Cosson, R.P., 2002. Induction of specific isoforms of metallothionein in mussel tissues after exposure to cadmium or mercury. Arch. Environ. Contam. Toxicol. 42, 36–42.

Geret, F., Jouan, A., Turpin, V., Bebianno, M.J., Cosson, R.P., 2002. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). Aquat. Living Resour. 15, 61–66.

Groppa, M.D., Benavides, M.P., 2008. Polyamines and abiotic stress: recent advances. Amino Acids 34, 35–45.

Groppa, M.D., Tomaro, M.L., Benavides, M.P., 2007. Polyamines and heavy metal stress: the antioxidant behavior of spermine in cadmium- and copper-treated wheat leaves. Biometals 20, 185–195.

Ha, H.C., Sirisoma, N.S., Kuppusamy, P., Zweier, J.L., Woster, P.M., Casero Jr., R.A., 1998. The natural polyamine spermine functions directly as a free radical scavenger. Proc. Natl. Acad. Sci. U. S. A. 95, 11140–11145.

Hamana, K., Niitsu, M., Samejima, K., Matsuzaki, S., 1991. Novel tetramines, pentamines and hexamines in sea urchin, sea cucumber, sea squirt and bivalves. Comp. Biochem. Physiol. B 100, 59–62.

Igarashi, K., Kashiwagi, K., 2000. Polyamines: mysterious modulators of cellular functions. Biochem. Biophys. Res. Commun. 271, 559–564.

Jan, T.K., Young, D.R., 1978. Determination of microgram amounts of some transition metals in seawater by methyl isobutyl ketone-nitric acid successive extraction and flameless atomic absorption spectrophotometry. Anal. Chem. 50, 1250–1253.

Kabra, P.M., Lee, H.K., Lubich, W.P., Marton, L.J., 1986. Solid-phase extraction and determination of dancyl-derivatives of unconjugated and acetylated polyamines by reverse-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. J. Chromatogr. B 380, 19–32.

Koukouzika, N., Dimitriadis, V.K., 2008. Aspects of the usefulness of five marine pollution biomarkers, with emphasis on MN and lipid content. Mar. Pollut. Bull. 56, 941–949.

Koutsogiannaki, S., Franzellitti, S., Fabbri, E., Kaloyianni, M., 2014. Oxidative stress parameters induced by exposure to either cadmium or 17β-estradiol on *Mytilus galloprovincialis* hemocytes. The role of signaling molecules. Aquat. Toxicol. 146, 186–195.

Lockwood, B.L., Somero, G.N., 2011. Transcriptomic responses to salinity stress in invasive and native blue mussels (genus *Mytilus*). Mol. Ecol. 20, 517–529.

Lovett, D.L., Watts, S.A., 1995. Changes in polyamine levels in response to acclimation salinity in gills of the blue crab *Callinectes sapidus* Raathbun. Comp. Biochem. Physiol. B110, 115–119.

Luo, W., Lu, Y., Wang, T., Kong, P., Jiao, W., Hu, W., Jia, J., Naile, J.E., Khim, J.S., Giesy, J.P., 2013. Environmental concentrations and bioaccumulations of cadmium and zinc in coastal watersheds along the Chinese Northern Bohai and Yellow Seas. Environ. Toxicol. Chem. 32, 831–840.

Matkovics, B., Kecskemeti, V., Varga, S.I., Novak, Z., Kertesz, Z., 1993. Antioxidant properties of di- and polyamines. Comp. Biochem. Physiol. B 104, 475–479.

Moore, M.N., 1985. Cellular responses to pollutants. Mar. Pollut. Bull. 16, 134–139.

Orlandini, G., Reali, N., Soldi, M.E., Bacciottini, F., Viviani, R., Casti, A., 1989. Effect of temperature and diet on polyamine concentrations of the European sea bass (*Dicentrarchus labrax* L.). Comp. Biochem. Physiol. B94, 581–585.

Outinen, K., Vuorela, P., Hinkkanen, R., Hiltunen, R., Vuorela, H., 1995. Optimization of the HPLC analysis of biogenic amines in *Peucedanun palustre* plants and cell culture lines. Planta Med. 61, 259–263.

Pegg, A.E., 2006. Regulation of ornithine decarboxylase. J. Biol. Chem. 281, 14529–14532.

Pegg, A.E., 2008. Spermidine/spermine-N^1-acetyltransferase: a key metabolic regulator. Am. J. Physiol. Endocrinol. Metab. 294, E995–E1010.

Perez-Leal, O., Merali, S., 2012. Regulation of polyamine metabolism by translational control. Amino Acids 42, 611–617.

Pytharopoulou, S., Grintzalis, K., Sazakli, E., Leotsinidis, M., Georgiou, C.D., Kalpaxis, D.L., 2011. Translational responses and oxidative stress of mussels experimentally exposed to Hg, Cu and Cd: one pattern does not fit at all. Aquat. Toxicol. 105, 157–165.

Pytharopoulou, S., Kournoutou, G.G., Leotsinidis, M., Georgiou, C.D., Kalpaxis, D.L., 2013. Cadmium versus copper toxicity: Insights from an integrated dissection of protein synthesis pathway in the digestive gland of mussel *Mytilus galloprovincialis*. J. Hazard. Mater. 260, 263–271.

Regoli, F., 1992. Lysosomal responses as sensitive stress index in biomonitoring heavy metal pollution. Mar. Ecol. Prog. Ser. 84, 63–69.

Rider, J.E., Hacker, A., Mackintosh, C.A., Pegg, A.E., Woster, P.M., Casero Jr., R.A., 2007. Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. Amino Acids 33, 231–240.

Seiler, N., 1987. Functions of polyamine acetylation. Can. J. Physiol. Pharmacol. 65, 2024–2035.

Serrano-Martinez, F., Casas, J.L., 2011. Effects of extended exposure to cadmium and subsequent recovery period on growth, antioxidant status and polyamine pattern in vitro cultured carnation. Physiol. Mol. Biol. Plants 17, 327–338.

Smirnova, O.A., Isaguliants, M.G., Hyvonen, M.T., Keinanen, T.A., Tunitskaya, V.L., Vepsalainen, J., Alhonen, L., Kochetkov, S.N., Ivanov, A.V., 2012. Chemically induced oxidative stress increases polyamine levels by activating the transcription of ornithine decarboxylase and spermidine/spermine-N^1-acetyltransferase in human hepatoma HUH7 cells. Biochimie 94, 1876–1883.

Stien, X., Percic, P., Gnassia-Barelli, M., Roméo, M., Laufaurie, M., 1998. Evaluation of biomarkers in caged fishes and mussels to assess the quality of waters in a bay of the NW Mediterranian Sea. Environ. Pollut. 99, 339–345.

Tzirogiannis, K.N., Panoutsopoulos, G.I., Demonakou, M.D., Papadimas, G.K., Kondyli, V.G., Kourentzi, K.T., Hereti, R.I., Mykoniatis, M.G., 2004. The hepatoprotective effect of putrescine against cadmium-induced acute liver injury. Arch. Toxicol. 78, 321–329.

Viarengo, A., Palmero, S., Zanicchi, G., Cappelli, R., Orunesu, M., 1985. Role of metallothioneins in Cu and Cd accumulation and elimination in the gill and digestive gland cells of *Mytilus galloprovincialis* Lam. Mar. Environ. Res. 16, 23–36.

Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic mollusks. Mar. Environ. Res. 44, 69–84.

Vouras, C., Dailianis, S., 2012. Evidence for phosphatidylinositol-3-OH-kinase (PI3-kinase) involvement in Cd-mediated oxidative effects on hemocytes of mussels. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 155, 587–593.

Wang, M.H., Wang, G.Z., 2009. Biochemical response of the copepod *Tigriopus japonicus* Mori experimentally exposed to cadmium. Arch. Environ. Contam. Toxicol. 57, 707–717.

Wimalasekera, R., Tebartz, F., Scherer, G.F.E., 2011. Polyamine, polyamine oxidases and nitric oxide in development, abiotic and biotic stresses. Plant Sci. 181, 593–603.

Xu, X., Shi, G., Jia, R., 2011. Changes of polyamine levels in roots of *Sagittaria sagittifolia* L. under copper stress. Environ. Sci. Pollut. Res. 19, 2973–2982.

Yang, H., Shi, G., Wang, H., Xu, Q., 2010. Involvement of polyamines in adaptation of *Potamogeton crispus* L. to cadmium stress. Aquat. Toxicol. 100, 282–288.

Zamora, R., Alaiz, M., Hidalgo, F.J., 1997. Feed-back inhibition of oxidative stress by oxidized lipid/amino acid reaction products. Biochemistry 36, 15765–15771.

Zappia, V., Porta, R., Carteni-Farina, M., De Rosa, M., Gambacorta, A., 1978. Polyamine distribution in eukaryotes: occurrence of sym-nor-spermidine and sym-nor-spermine in arthropods. FEBS Lett. 94, 161–165.